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ATTY.'S DOCKET: LANDEGREN=1A

In re Application of:	)	Art Unit: 1637
	)	
Ulf LANDEGREN et al	)	Examiner: S. Chunduru
	)	
Appln. No.: 09/785,657	)	Washington, D.C.
	)	
Date Filed: February 20, 2001	)	Confirmation No. 5356
	)	
For: METHODS AND KITS FOR PROXIMITY PROBING	)	March 26, 2005

DECLARATION UNDER 37 CFR §1.132

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Sir:

I, Ulf LANDEGREN, hereby declare and state as follows:

I am a coinventor of the above-identified application and my educational and professional experience is presented in the curriculum vitae attached hereto.

It is my understanding that the claims of the above-identified application have been rejected under 35 U.S.C. §103(a) as being unpatentable/obvious over my own prior patent publication, WO 97/00446, as the primary reference and Ebersole et al., WO 97/32044, as the secondary reference.

The experiments described below, which demonstrate the unexpected results obtained by the liquid phase (solution) based proximity probe detection method (hereinafter referred to as the homogeneous assay or Prox II) of the present invention claimed in the

above-identified application in comparison to the solid phase anchored proximity probe detection method of WO 97/00446 (hereinafter referred to as the solid phase assay or Prox I), were conducted by me or under my direct supervision, and I can attest of my own personal knowledge that all the results reported herein are true and accurate.

A proximity probe pair consisting of SELEX-aptamer derived binding moieties with extended oligonucleotides for proximity dependent ligation was used to detect the analyte, Platelet derived growth factor BB (PDGF-BB). For the solid phase anchored assay (Prox I), the analyte PDGF-BB and the proximity probes bound thereto were first immobilized by an antibody specific for the analyte and coupled to the solid support. Excess amounts of proximity probes were then removed by washing the support. The proximity probes bound to the immobilized analyte were then ligated and the ligation product subsequently detected by real-time PCR.

In the homogeneous assay (Prox II), the proximity probes were mixed with the sample containing analyte, equilibrated, and then ligated together without the removal of excess probes or immobilization of the target analyte. The ligation product was detected and quantified by real-time PCR.

The same target antigen and proximity probe pair were used in both experiments. Both assays were conducted under conditions optimized for maximum sensitivity. Figure 1 presented below schematically illustrates the solid phase assay (Prox I) compared to the homogeneous proximity probe assay (Prox II).

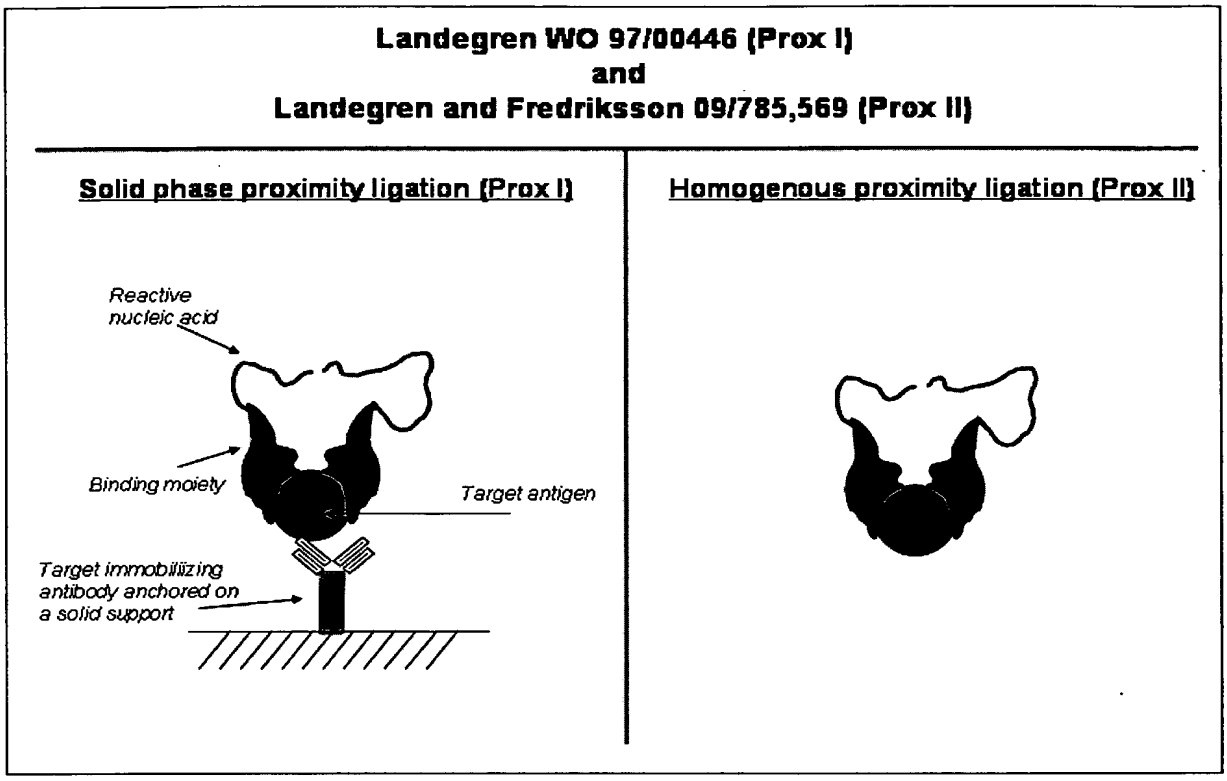

FIG. 1

The assays were performed as follows:

Solid phase anchored assay (Prox I)

Detection of PDGF-BB by solid phase proximity ligation. Five hundred nanogram of an anti-PDGF antiserum immunoglobulin fraction in 20 μl was added to optical tubes for real-time PCR, followed by blocking with 1% BSA. Up to 200 μl of sample was then incubated together with 5 nM of proximity probes A1 and A2. Unbound probes were removed by washes using a multichannel pipette (8 times with 3 flushes of 200 μl PBS 0.02% SDS, PBS and finally H_2O). A ligation mix (50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 0.15 mM ATP, 50 nM connector oligonucleotide, 2 U T4 DNA ligase in 20 μl) was added and the

reactions were kept at 30°C for 5 minutes followed by 20 minutes at 80°C. Next, a PCR mix was added to a final volume of 50 μl, containing ROX internal fluorescence standard, 0.1 mM dNTPs, 0.2 μM primers (forward: 5'-atgtggtctat gtcgtcgttcg-3', reverse: 5'-tgagtaagaacagcgcg cat-3'), 50 nM probe for the 5' nuclease assay, 1 unit AmpliTaq Gold polymerase. After a five minute ligation reaction at room temperature, the reactions were transferred to the real-time PCR instrument for temperature cycling; 95°C for 10 minutes and then 95°C 15 seconds and 60°C 60 seconds, repeated 45 times (ABI PRISM 7700). The number of ligation products was calculated from a standard curve of diluted amplicons.

Homogeneous liquid phase assay (Prox II)

One μl aliquots, containing varied amounts of PDGF-BB diluted in 137 mM NaCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4 pH 7.4, 2.7 mM KCl, 1 mM $MgCl_2$, 1% bovine serum albumin (BSA), were added to optical tubes (Applied Biosystems, Foster City, CA) containing 20 pM of the proximity probes A1 and A2 in a total volume of five μl 50 mM KCl, 10 mM Tris-HCl pH 8.3, 3.3 mM $MgCl_2$, and 0.1% BSA. Upon addition of the combined mix for ligation and amplification the samples contained 50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 0.4 units T4 DNA ligase, 400 nM connector oligonucleotide, 80 μM ATP, ROX internal fluorescence standard, 0.2 mM dNTPs, 0.5 μM primers, 50 nM probe for the 5' nuclease assay, and 1.5 units AmpliTaq Gold polymerase (ABI) in 50 μl. After a five minute ligation reaction at room temperature, the reactions were transferred to the real-time PCR instrument for temperature cycling; 95°C for 10 minutes and then 95°C 15 seconds and 60°C 60 seconds,

repeated 45 times (ABI PRISM 7700). The number of ligation products was calculated from a standard curve of diluted amplicons.

Results

As can be seen in Figure 2 below, which is a comparison between the solid phase anchored and the homogeneous proximity ligation assays for the detection of PDGF-BB as analyte, the homogeneous proximity ligation assay (Prox I) can detect about 10 times lower amounts of the target analyte than the solid phase anchored version of the assay. Signal to noise ratios on the Y-axis denote the number of ligation reactions derived from the reactions with target analyte present (signal) divided by the number of ligations derived from a reaction with no target present (noise). Furthermore, not only is the homogeneous proximity ligation assay of the present invention (Prox I) a simpler assay to perform than the solid phase anchored assay (Prox II) since it requires no washing steps, but it also consumes over 10,000 times less of the probes, $100x10^{-18}$ moles (attomoles) compared to $1x10^{-12}$ moles (picomoles).

Since the homogeneous proximity ligation assay contains no washing steps, it was initially expected that the homogeneous proximity ligation assay would produce too many background ligation events, far surpassing the signals from target binding events, and therefore would result in an insensitive assay. Surprisingly, however, the background in the homogeneous ligation assay was discovered to be very low since the reactivity of the nucleic acids at a low concentration in solution was found to be low in comparison to those bound to the target analyte. Target analyte bound proximity probes gain greatly in reactivity due to

the enhanced effective concentration of the reactive nucleic acids in the local immediate environment surrounding the analyte.

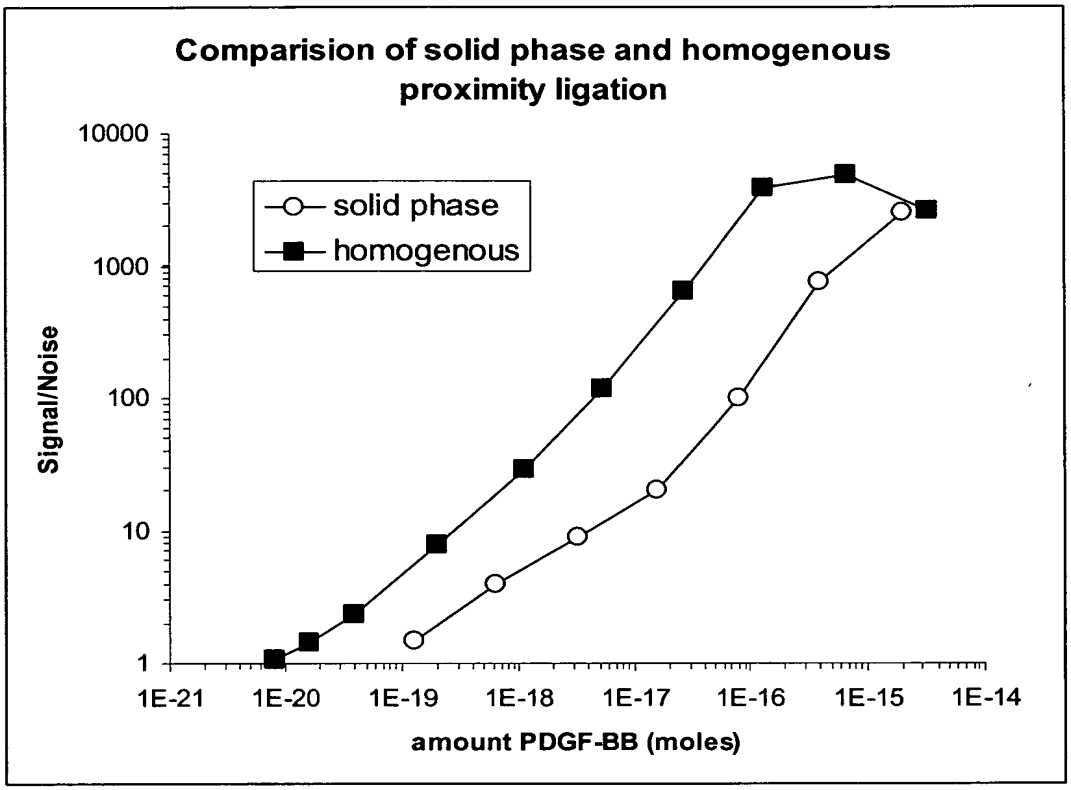

Fig. 2

In conclusion, the inventors of the present above-identified application have discovered unexpectedly superior results for the homogeneous proximity ligation assay (Prox II) over what was obtained with the solid phase proximity ligation assay (Prox I) of WO 97/00446. The unexpectedly superior results demonstrate that the homogeneous proximity ligation assay, compared to the solid phase proximity ligation assay, has a significantly lower limit of detection for the analyte while requiring much less amounts (by many orders of magnitude)

of proximity probes, thereby lowering reagent consumption, as well as being easier and more rapid to perform because it omits at least the extra steps of immobilizing antibodies/analyte to a solid support and of washing to remove unbound proximity probes found in the solid phase assay.

The undersigned declares further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

March 26, 2005
Date

Ulf LANDEGREN

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CURRICULUM VITAE

ULF LANDEGREN
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Marital Status: Married. Three children, born 1984, 1986, and 1987
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EDUCATION

1984-1988 Postdoctoral studies in Molecular Biology at California Institute of Technology. Supervisor: Dr. Leroy Hood
1979-1984 Graduate studies in Cellular Immunology at Uppsala Biomedical Center, Sweden. Advisor: Dr. Hans Wigzell. PhD 1984.
1973-1979 School of Medicine, University of Uppsala. MD 1979.
1972-1973 Mathematics, one year. University of Uppsala, 1972

PROFESSIONAL HISTORY

1996- Professor of Molecular Medicine at Uppsala University
1993-1996 Associate Professor of Medical Genetics
1991-1993 Assistant Professor of Medical Genetics
1989-1991 Scientist at the Department of Medical Genetics, Biomedical Center of Uppsala, Sweden
1988-1989 Coordinator of the DNA Diagnostics Program, Division of Biology, California Institute of Technology (Caltech), Pasadena, California
1984-1988 Research Fellow, Division of Biology, Caltech
1979-1984 Graduate Student and Teaching Assistant, Department of Immunology, Biomedical Center of Uppsala, Sweden
1977-1984 Assistant Medical Doctor at Departments of Medicine, Surgery, and Gynecology for a total of around one and a half years
1971-1972 Laboratory Assistant, Department of Genetics and Plant Breeding, Agricultural College of Sweden, Uppsala

TEACHING

1996-2002 Member of the committee for education at the medical faculty of Uppsala.
1989-1995 Coordinator of the Medical Genetics courses at the School of Medicine, University of Uppsala
1992-present Organizer and coorganizer of six advanced international theoretical and practical courses in molecular medicine, and five corresponding courses for clinicians
1979 - 1984 Lectures and supervision of lab work during courses in Immunology and Medical Immunology at Uppsala University

COMMISSIONS OF TRUST

2004 – present Founder, board member, and chairman of the scientific board of Olink AB, Uppsala Sweden
2004 – present Member of the scientific advisory board of the Max Planck Institute for Molecular Genetics, Berlin, Germany
2004 – present Member of the board of the Center for Science and Technology Studies, Uppsala University
2004 – present Visting senior scientist at the lab of Dr Yoshihide Hayashizaki, the RIKEN Institute, Yokohama, Japan

2003 – present Coordinator of the EU Frameworkprogram 6 Integrated Project "MolTools" (www.moltools.org)

2003 – 2004 Chair of the Life Science Technology committee of the Swedish Strategic Foundation committee for Future Research Leader awards

2003 - present Member, Swedish Research Council committee on EU Research in Life Science, Genomics and Biotechnology for Health

2002 – present Coordinator of biotechnology at Uppsala University

2001 – present Cofounder and chairman of the scientific advisory board of Pal Allele BioScience, South San Francisco

2001 – present Member of the Royal Society of Sciences, Sweden

1999 - 2001 Director of the Uppsala node of the Wallenberg Consortium North for Functional Genomics

1999 – present Swedish representative in a network for functional genomics, supported by the European Science Foundation

1998 - present Member of the scientific advisory board of Sequenom, San Diego, CA, USA

1998 – present Member of the Human Genome Organization (HUGO) committee on intellectual property rights

1998 - 1999 Coorganizer of a series of international meetings on single-nucleotide polymorphisms and complex genome analysis

1997 - 2001 Member of the board of directors of Professional Genetics Laboratory AB

1995 - 1999 Coorganizer of a semiannual series of international meetings on Mutation Detection

1995 - 2003 Member of the Swedish Research Council for Medicine, Review committee on Cell Biology I

1993 – 1996 Consultant in Molecular Biology for Pharmacia Biotech, exploratory research

1993 - 2001 Consultant in Molecular Medicine at the Department of Medicine, Uppsala Academic Hospital

1991 - present Member of the Human Genome Organization (HUGO)

Member of the board of editors of **Genome Research,** published by Cold Spring Harbor Laboratory Press until 2004, **Human Mutation**, Published by John Wiley and sons, **Mutation Research Genomics**, published by Elsevier, **Compative and Functional Genomics**, Published by Wiley-VCH, and **er-Molecular Medicine** - an on-line journal

Reviewer of manuscripts also for **Science, Nature Genetics, Nature Biotechnology, Nature Medicine, Nucleic Acids Research, Proc Natl Acad Sci USA, Clin Chem, Biotechniques, Human Genetics,** etc

Member of committees evaluating research in Germany. Reviewer of grant applications for the Swedish Research Council for Medicine, the Swedish Strategic Foundation, the EU, Wellcome Foundation, US Department of Agriculture, and the research councils of Italy, Israel, The Netherlands, Norway etc

STIPENDS

The Eric K. Fernström award in 1993
The Bertil Åberg Biotechnology award in 1993
The Procordia Foundation, twice
The Nilsson-Ehle Foundation, twice
The Borgströms Foundation, four times
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Mathias Howell

PUBLICATIONS, Research

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58. Melin J, Johansson H, Söderberg O, Nikolajeff F, Nilsson M, Landegren U, Jarvius J. A disposable microfluidic platform enabling actuatation, cell culture, and sensitive fluorescence detection. Submitted

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60. Stenberg J, Nilsson M, Landegren U. ProbeMaker: a framework for construction of oligonucleotide sets. Submitted

61. Söderberg O, Gullberg M, Emilson M, Bahram F, Larsson LG, Landegren U. Localized detection of proteins *in situ* using proximity ligation. In preparation

REVIEWS AND BOOK CHAPTERS

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